Published in final edited form as:

Biochem Biophys Res Commun. 2010 March 19; 393(4): 678–681. doi:10.1016/j.bbrc.2010.02.055.

UDP-GlcNAc: Glycoprotein N-Acetylglucosamine-1-Phosphotransferase Mediates the Initial Step in the Formation of the Methylphosphomannosyl Residues on the High Mannose Oligosaccharides of *Dictyostelium Discoideum* Glycoproteins

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Abstract

The *Dictyostelium discoideum* gene *gpt1* encodes a protein XP_638036 with sequence similarity to the α/β subunits of mammalian UDP-GlcNAc:Glycoprotein N-acetylglucosamine-1-phosphotransferase. We now demonstrate that extracts of *D. discoideum* clones with mutations in this gene transfer GlcNAc-P from UDP-GlcNAc to mannose residues at less than 5% the wild type value. Further, the lysosomal hydrolases of these mutant clones fail to bind to a cation-independent mannose 6-phosphate receptor affinity column, indicating a lack of methylphosphomannosyl residues on the high mannose oligosaccharides of these proteins. We conclude that the *gpt1* gene product catalyzes the initial step in the formation of methylphosphomannosyl residues on *D. discoideum* lysosomal hydrolases.

Keywords

Dictyostelium discoideum; lysosomal hydrolases; methylphosphomannosyl residues; GlcNAc-1-phosphotransferase

Introduction

Previously we established that the lysosomal enzymes of the slime mold *Dictyostelium discoideum* contain mannose 6-phosphate (Man-6-P) residues on their high mannose oligosaccharides that are diesterified to methyl groups [1]. We also found that this organism has an active UDP-GlcNAc:Glycoprotein N-Acetylglucosamine-1-Phosphotransferase (GlcNAc-1-phosphotransferase) that transfers GlcNAc-P to mannose residues of high mannose oligosaccharides to form GlcNAc-P-Man diesters [2]. However, the potential role of this transferase in the formation of the methylphosphomannosyl residues was not established.

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Subsequently, Freeze and coworkers reported that extracts of *D. discoideum* utilized S-adenosylmethionine as a donor for the transfer of a methyl group to Man-6-P [3]. In contrast, GlcNAc-P-Man was inactive as an acceptor in this assay, presumably due to an inability to convert this substrate to Man-6-P by excision of the GlcNAc. This raised the possibility that a GlcNAc-1-phosphotransferase is not involved in the formation of the methylphosphomannosyl residues.

In this study we identify the gene that encodes the *D. discoideum* GlcNAc-1-phosphotransferase and show that it is essential for the formation of the methylphosphomannosyl residues present in the lysosomal hydrolases. This allows us to propose a biochemical scheme for the formation of this unusual modification.

Material and methods

Materials

Dictyostelium discoideum strain TL276 (V31811, DBS0266153) and TL277 (V31807, DBS0266149) were from Dr. E.C. Cox (Princeton). These strains were isolated in a screen for abnormal growth or development of strain A×4 subjected to insertional mutagenesis using DpnII and pBSR1 [4], and we have confirmed a slow growth phenotype in axenic media and morphological differences during development (unpublished data). After digestion of genomic DNA of TL276 with RsaI and religation, sequencing from the T7 promoter element of pBSR1 yielded sequence data indicating insertion at the DpnII site beginning at nt 3189 (relative to the start codon of DDB0304518 [gpt1] at dictybase.org). This corresponds to a predicted interruption of Gpt1 after Asp993 of the primary translation product, within a highly conserved region of the β -subunit. After digestion of genomic DNA of TL277 with EcoRI and religation, sequencing from the T7 promoter yielded sequence indicative of insertion of pBSR1 at the same position [12]. UDP-[6-³H]GlcNAc (20 Ci/mmol) was purchased from PerkinElmer (Waltham, MA). α -methylmannoside, QAE-Sephadex, and lysosomal enzyme substrates (4-methylumbelliferyl α -D-mannopyranoside, 4-methylumbelliferyl β -D-galactopyranoside, and 4-methylumbelliferyl N-acetyl- β -D-glucosaminide) were from Sigma (St. Louis, MO). The CI-MPR was purified from calf liver and immobilized as previously reported [5]. All other reagents were of the highest quality available and were purchased from Sigma or Fisher.

Dictyostelium discoideum lysate preparation

Cells were grown in axenic growth medium (HL-5) to $5\text{--}10 \times 10^6/\text{ml}$ in shaking flasks. 10^9 cells were collected by centrifugation at $1000 g \times 1 \text{ min}$, resuspended in ice-cold TBS [10 mM Tris-HCl (pH 7.4), 150 mM NaCl], and 10^8 -cell aliquots were centrifuged again. The supernatants were aspirated and cell pellets were frozen at -80°C .

100 μl cell pellets were resuspended in 900 μl PBST (PBS+1% Triton X-100) and sonicated on ice for 10 sec. The cell lysates were then incubated on ice for 15 min, centrifuged at 14,000 rpm for 15 min at 4°C , and the supernatant fractions collected. The protein concentrations in the supernatants were determined by Bradford assay. The supernatants were used for GlcNAc-1-phosphotransferase assay, lysosomal enzyme assays and CI-MPR affinity column analysis.

GlcNAc-1-phosphotransferase assay

The reaction mixture contained 50 mM α -methylmannoside, 50 mM Tris-HCl pH 7.4, 10 mM MgCl_2 , 10 mM MnCl_2 , 2 mM ATP, 75 μM UDP-[³H]GlcNAc (1 μCi), 2 mg/ml BSA, and 300 μg *D. discoideum* cell lysates in a final volume of 50 μl . Incubations were for 1 hour at 37°C . Reactions were terminated by addition of 1 ml 5 mM EDTA, pH 8.0. The sample was applied to a 1 ml column of QAE-Sephadex equilibrated with 2 mM Tris base. The column was washed

with 1 ml 2 mM Tris base 5 times and the [^3H]GlcNAc-P- α -methylmannoside product eluted with 1 ml 30 mM NaCl in 2 mM Tris base 5 times. The incorporated [^3H]GlcNAc-P was determined by scintillation counting following addition of 10 ml EcoLite scintillation fluid (MP Biomedicals Inc., Irvine, CA). The GlcNAc-1-phosphotransferase activity was expressed as picomoles of GlcNAc-1-P transferred per hour per mg protein. Reactions incubated in the absence of α -methylmannoside served as the background values and were subtracted from the complete reactions.

Lysosomal enzyme assays

Lysosomal hydrolase activities were determined by fluorometric enzyme assays as described previously [6]. In brief, samples were incubated with 4-methylumbelliferyl-coupled specific substrates in a 50 mM citrate buffer containing 0.5% Triton X-100, pH 4.5, at 37°C. α -Mannosidase was assayed with 5 mM 4-methylumbelliferyl α -D-mannopyranoside, β -Galactosidase was assayed with 5 mM 4-methylumbelliferyl β -D-galactopyranoside, and β -hexosaminidase was with 1 mM 4-methylumbelliferyl N-acetyl- β -D-glucosaminide. Reactions were stopped by addition of 1 ml 0.1 M glycine-NaOH solution, pH 10.3, and the fluorescence was read at 495 nm.

CI-MPR Affinity Chromatography

D. discoideum cell lysates (300 μg protein) were diluted in CI-MPR column buffer (50 mM imidazole, pH 6.5, 0.15M NaCl, and 0.05% TX-100) to a total volume of 1 ml and loaded on a CI-MPR affinity column (0.5 mg CI-MPR/ml Sepharose) prepared as described previously [5]. The columns were washed with 7 ml of column buffer and then eluted with 5 ml of column buffer containing 10 mM Glucose 6-phosphate (Glc-6-P), and finally with 5 ml of column buffer containing 10 mM Man-6-P. One ml fractions were collected and assayed for lysosomal enzyme activity as described above.

Results

Identification of the gene encoding GlcNAc-1-phosphotransferase in *D. discoideum*

A blast search of *D. discoideum* genome sequences identified a hypothetical protein (XP_638036) of 1238 amino acids, of which 335 are identical to those present in the α/β subunits of the human GlcNAc-1-phosphotransferase (see supplemental Fig. 1). The α/β subunits of the human enzyme are encoded by a single gene that produces a precursor protein that undergoes a proteolytic clip to generate the α and β subunits [7]. The holoenzyme is an $\alpha_2\beta_2\gamma_2$ hexamer with the γ subunits encoded by a separate gene [8, 9]. Interestingly, we were unable to identify a gene homologous to the γ gene in the *D. discoideum* genome.

Fig. 1 shows a schematic view of the human α/β subunits. The α subunit contains three regions that are shared with other proteins, the bacterial capsule biosynthesis protein-like domain, the two Notch repeats, and the DMAP binding domain [7,10]. The first domain is present in a number of proteins postulated to be glycosyltransferases that mediate capsule formation in bacteria and fungi [11]. The DMAP binding domain has been proposed to be a protein-protein interacting domain [12]. The *D. discoideum* gene exhibits strong identity to the bacterial capsule biosynthesis protein-like domain (54%) and the Notch repeat domain (49%) of the human α subunit. It lacks the DMAP binding domain, which is replaced by a non-conserved sequence with low complexity. Its C-terminal region has 31% identity with the β subunit.

Since the α/β subunits of human GlcNAc-1-phosphotransferase contain the catalytic activity of the enzyme, XP-638036 was a good candidate for mediating this activity in *D. discoideum* cells. To determine if this was the case, we turned to two mutant strains (T1276 and TL277) that had been picked up in a high-throughput REMI screen that looked for altered

phenotypes of mutants in *D. discoideum* [4]. The TL276 and TL277 clones, which exhibited aberrant growth, were shown to have mutations in the gene *gpt1* which encodes the hypothetical protein XP_638036.

Lysates of A×3 wild type cells and the two mutants were incubated with 50 mM α -methylmannoside and 75 μ M UDP-[³H]GlcNAc for 1 hour and the transfer of [³H]GlcNAc to α -methylmannoside was measured as described in Experimental Procedures. Table I shows that the extracts of the two mutant cell lines had less than 5% the activity of the A×3 strain. We conclude that the hypothetical protein XP_638036 is indeed the GlcNAc-1-phosphotransferase in *D. discoideum*.

Protein XP_638036 is required for the formation of methylphosphomannosyl residues on lysosomal enzymes

The lysosomal enzymes of *D. discoideum* bind with high affinity to the cation-independent mannose 6-phosphate receptor (CI-MPR) via their methylphosphomannosyl residues [1,13–15]. If GlcNAc-1-phosphotransferase mediates the first step in the synthesis of the methylphosphomannosyl residues, we would predict that the lysosomal enzymes of the TL276 and TL277 mutants would be deficient in this modification. To test this, extracts of A×3, TL276, and TL277 cells were applied to a CI-MPR-affinity column and eluted sequentially with buffer alone, buffer containing 10 mM Glc-6-P and finally buffer with 10 mM Man-6-P. The various fractions were then assayed for three lysosomal hydrolases: α -mannosidase, β -galactosidase and β -hexosaminidase. Fig. 2 shows that essentially all of the three hydrolases of the A×3 cells bound efficiently to the column and were specifically eluted with the Man-6-P. By contrast, all of the activity of the TL276 and TL277 lysosomal hydrolases was recovered in the flow-through and buffer-wash fractions, indicating a total loss of binding to CI-MPR. This clearly shows that protein XP_638036 is essential for the synthesis of the methylphosphomannosyl residues on these lysosomal hydrolases.

Discussion

The data presented in this report combined with the finding of Freeze, et. al. [3] demonstrate that the synthesis of the Man-6-P-OCH₃ residues on the lysosomal hydrolases of *D. discoideum* occurs by the scheme shown in Fig. 3. The initial step is catalyzed by the product of the *gpt1* gene which transfers GlcNAc-1-P residues from the donor UDP-GlcNAc to specific mannoses on the N-linked high mannose glycans of the acceptors. This is followed by the cleavage of the GlcNAc residues by an “uncovering” enzyme yet to be identified. Finally, a S-adenosylmethionine-dependent methyltransferase acts on the Man-6-P monoesters to generate the Man-6-P-OCH₃ residues.

The evidence that the *gpt1* gene encodes the *D. discoideum* GlcNAc-1-phosphotransferase that mediates the first step in the scheme shown in Fig. 3 is two fold. First, two strains with mutations in this gene exhibit less than 5% of wild type activity in the standard GlcNAc-1-phosphotransferase assay using α -methylmannoside as acceptors. And second, the lysosomal hydrolases of these mutant strains fail to bind to a CI-MPR-affinity column whereas essentially all of these enzymes from the wild type strain bind and are specifically eluted with Man-6-P. This binding is known to be mediated by the Man-6-P-OCH₃ residues of the lysosomal hydrolases [13].

The GlcNAc-1-phosphotransferase of *D. discoideum* differs from the mammalian enzyme in that the α subunit lacks the DMAP binding domain. The DMAP binding domain has been postulated to be a protein-protein interaction domain and could potentially have a role in the recognition of the protein determinants on lysosomal hydrolase substrates [12]. While the *D. discoideum* GlcNAc-1-phosphotransferase lacks the ability to recognize the protein

determinant of mammalian lysosomal hydrolases [2], its ability to interact with *D. discoideum* hydrolases has not been studied. It is also of interest that the *D. discoideum* GlcNAc-1-phosphotransferase appears to lack the γ subunit that is present in the mammalian enzyme, based on the absence of γ subunit-related sequences in the genome (unpublished data). This subunit is required for the efficient phosphorylation of a subset of the lysosomal hydrolases by the mammalian enzymes [16]. Since the lysosomal hydrolases of wild type *D. discoideum* are well phosphorylated, the γ subunit seems to be dispensable in this species.

Finally, it is of note that the cellular content of the three lysosomal hydrolases measured in the experiments shown in Fig. 2 was much higher in the TL276 and TL277 extracts compared to the wild type A $\times$ 3 extract. The cells used in these experiments were harvested in the late vegetative stage when the A $\times$ 3 cells secrete much of their content of lysosomal hydrolases into the media [17]. In preliminary experiments, we have found that hydrolase secretion by the TL276 and TL277 cells is impaired when compared to A $\times$ 3 cells (unpublished data). This suggests that the methylphosphomannosyl residues may have a role in the sorting and/or secretion of lysosomal hydrolases in *D. discoideum*. The demonstration that the TL276 and TL277 strains are unable to synthesize this unusual modification will allow for studies to define the role of the Man-6-P-OCH₃ residues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The work was supported by National Institutes of Health Grant CA08759 (to S.K.), R01-GM-37539 (to C.W.) and training grant T32 HL007088 (to Y.Q.). We are grateful to Georgia Guan and Satoshi Sawai for retrieval of insertion sequence data from genomic DNA of strains TL276 and TL277. We also thank Jenny Lu for assistance with several experiments.

Abbreviations

<i>D. discoideum</i>	<i>Dictyostelium discoideum</i>
GlcNAc-1-phosphotransferase, UDP-GlcNAc	Glycoprotein N-Acetylglucosamine-1-Phosphotransferase
Man	mannose
GlcNAc	N-acetylglucosamine
CI-MPR	cation independent mannose 6-phosphate receptor

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Fig. 1. Schematic of *H. sapiens* GlcNAc-1-phosphotransferase α/β subunits vs. *D. discoideum* XP_638036. The human enzyme precursor is cleaved into two subunits- α and β . The identity of all domains between *H. sapiens* and *D. discoideum* is listed. Note that the DMAP binding domain is missing in the *D. discoideum* XP_638036. The position of Asp993 is indicated in the *D. discoideum* XP_638036.

Fig. 2.

Protein XP_638036 is required for adding Man-6-PO-CH₃ residues to lysosomal acid hydrolases in *D. discoideum*. Wild type A×3 and two mutants TL276 and TL277 cell lysates were loaded on a CI-MPR-affinity column and eluted with buffer, buffer with 10 mM Glc-6-P and buffer with 10 mM Man-6-P. Samples were collected in 1 ml fractions and assayed for α-mannosidase, β-galactosidase and β-hexosaminidase.

Fig. 3.

Scheme for Man-6-P-OCH₃ synthesis. The scheme indicates that the terminal Man on the 3'-arm is removed prior to addition of GlcNAc-P to the penultimate Man. This has been shown to be the case in mammalian cells, but not directly demonstrated in *D. discoideum*.

Table 1

GlcNAc-1-phosphotransferase activity

<i>D. discoideum</i> strains	GlcNAc-1-phosphotransferase Activity (pmol/hr/mg)
A×3	1303±316 (n=3)
TL276	55±36 (n=2)
TL277	41±27 (n=2)